THE GENETICS OF ARTEMIA SALINA. I. THE REPRODUCTIVE CYCLE[1]

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The brine shrimp *Artemia salina* is a branchiopod crustacean found in saline lakes and the evaporating ponds of commercial salt works. It is of interest to geneticists because amphigonic races have been reported to be diploid ($2n = 42$) or tetraploid. Parthenogenetic races have been found to be diploid, triploid, tetraploid, pentaploid, and octaploid. Barigozzi (1957) has reviewed the cytological studies of these races. No previous attempt has been made to analyze traits which are governed by a single locus.

Artemia is easily cultured in the laboratory because it is resistant to environmental stresses. In a study of a California salt pond containing brine shrimp, Carpelan (1957) found diurnal changes of 12° C. in the water temperature in August. Provasoli and Shiraishi (1959) raised nauplii to adulthood in a sterile medium. Lochhead (1941) reported that females reproduce viviparously or oviparously. The thick-shelled egg contains a blastula and withstands desiccation for as long as 15 years. Therefore, mutant stocks might be conveniently stored in the form of thick-shelled eggs (cysts) without need of repeated subculture.

Because the shrimp is transparent, the effect of genes upon cellular differentiation may be studied throughout the development of one individual. Weisz (1946) has pointed out the advantages of studying morphogenesis in this primitive crustacean which has nineteen body segments but few specialized structures to obscure the principles of development. He has stated (in 1947, p. 87) that the "... histological sequences are found to be governed by a continuous overall pattern of metameric development, precisely defined in relative time and in space. ..."

In 1959, the author began a study of *Artemia* in the hope of developing a method for raising shrimp through many generations in pedigreed cultures. This paper describes a successful culture method and a series of experiments which test for sperm storage by the female and reproduction by parthenogenesis, paedogenesis, and pseudogamy.

Materials and Methods

The cysts of the California race were collected from salt works on San Francisco Bay; those of the Utah race were from Great Salt Lake. The dried cysts are routinely stored in glass bottles and hatched in sea water. In 24 to 36 hours, the shells burst and each embryo emerges enclosed within a transparent membrane. In another eight hours, the embryo hatches out as a free-swimming nauplius. The

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nauplii are transferred directly to the *culture medium* made by adding 50 grams of NaCl to one liter of filtered sea water.

All cultures are maintained in 5 cc. of culture medium in shell vials 21 mm. in diameter and 70 mm. high. A *standard yeast suspension* (SYS) is made by mixing 1 cc. of dry Brewer's yeast with 9 cc. of medium. It is dispensed into the shell vials by means of a pipette once per week according to this *standard feeding schedule:*

First day. Nauplii are separated from their parents (in the case of laboratory stocks) or from the shells (if the cysts have been collected from salt ponds). One to three nauplii are put in each vial, 0.05 cc. of SYS is added, and the vial is tightly corked.

Eighth day. Again, 0.05 cc. of SYS is added to each vial, irrespective of the number of surviving metanauplii.

Fifteenth day. Five-one hundredths cc. of SYS is added for every shrimp present in the vial. Many will have reached sexual maturity. Males are easily identified by their larger antennae.

Twenty-second day. Five-one hundredths cc. of SYS is added for every shrimp in the vial. Mated pairs may have produced a brood of nauplii.

Twenty-ninth day, etc. The weekly feedings of 0.05 cc. of SYS per adult are continued.

The stocks are maintained at room temperature (20°–28° C.) in a room illuminated during the day by artificial light. No attempt is made to aerate the medium nor to remove waste materials. When raised by this method, more than half of the nauplii born to laboratory stocks reach sexual maturity and a few shrimp have reached the age of nine months. Mated females give birth to free-swimming nauplii; virgin females release transparent thin-shelled eggs which sink to the bottom of the vial and do not hatch. Opaque thick-shelled cysts are not produced unless the culture method is modified.

Results and Discussion

1. *Effect of food quantity upon viability and fertility*

The standard feeding schedule described above was adopted because shrimp can reach maturity if the standard amount of food is either doubled or cut in half; *i.e.*, it allows margin for measurement errors. It also results in optimum viability, as the following experiment demonstrates. One first-instar California nauplius was placed in each of 276 vials containing 5 cc. of culture medium. All nauplii received the standard amount of SYS until the fifteenth day when the 198 survivors were divided into three equal groups of 66. Each group then was put on a different regimen: 0.025 cc., 0.05 cc., or 0.10 cc. of SYS per shrimp each week. The data in Table I indicate that viability was highest in those shrimp receiving the standard quantity (0.05 cc.) of SYS. Females were examined by transmitted light under a binocular microscope to see if opaque yolk granules were in the eggs. Because vitellogenesis was first seen in females receiving 0.1 cc. of SYS per week (Table II), we may conclude that females fed twice the standard amount mature faster than those on the standard schedule.

TABLE I

Effect of quantity of food upon viability

Amount of SYS each week	Number of living shrimp				
	Age (days)				
	15	22	29	36	57
0.025 cc.	66	41	30	24	14
0.05 cc.	66	44	38	34	28
0.10 cc.	66	44	38	35	20

2. *Relation of age and fertility*

When different males were mated successively to a fertile female, none was found to be sterile. But many females consistently produced either small broods of nauplii or broods of thin-shelled eggs which did not hatch. About one-half of both the wild and the mutant females had high fertility records like those shown in Table III. Note that the number of nauplii per brood is not correlated with the age of the female. The brood size may be influenced by uncontrolled factors such as the type of bacterial flora in the vial or the time of feeding in relation to the reproductive cycle. In another experiment, four pairs of shrimp, all of which were over five months of age, produced broods of normal size (39–76 nauplii) and the nauplii had normal viability. Fertility evidently does not decline throughout the first five months of life.

3. *Tests for parthenogenesis and paedogenesis*

To test for parthenogenesis, 20 immature females of the Utah race and 80 of the California race were isolated, one in each vial. They did not produce nauplii but laid broods of transparent eggs every four or five days. A control group was kept for the same period of one month in the presence of males. All of the 20 Utah controls and 64 of the 80 California controls gave birth to nauplii.

More than 200 females of each race have been isolated and parthenogenesis has never been observed. Nauplii are born three to five days after the females have

TABLE II

Effect of quantity of food upon fertility

Amount of SYS each week	Females showing vitellogenesis/total females			
	Age (days)			
	22	29	36	57
0.025 cc.	0/20	0/15	0/10	3/5
0.05 cc.	1/22	6/18	11/16	11/12
0.10 cc.	10/22	17/20	16/18	11/12

mated and at no other time. All attempts to hatch the eggs of virgin shrimp have failed. These findings are in agreement with those of Lochhead (1941), who found that fertilization was essential for reproduction in the California race. However, both Jensen (1918) and Relyea (1937) reported that the Utah race could reproduce parthenogenetically. These two authors did not provide sufficiently detailed accounts of their experiments to permit an attempt to replicate them in this present study.

In order to test for paedogenesis, California nauplii were allowed to grow to adulthood in the presence of their mother. In the fourteen cultures observed, the male nauplii were unable to fertilize their mother or their female sibs until they reached the tenth instar of Heath (1924) when their antennae took on the adult shape, enabling them to clasp the female. In 24 cultures, California fe-

TABLE III

Relation of age and fertility in three Utah females

Female	Number of nauplii in brood								
	Age 4–7 weeks				Age 8–12 weeks				
A	9	19	16	31	70	38	30		
B	17	45	15	56	20	19	36	41	70
C	69	3	42	58	71	92	2		

males raised with their fathers also failed to reproduce until they reached adulthood and vitellogenesis was present. Hundreds of nauplii from both races have been paired during routine maintenance of stock cultures, with no evidence of paedogenesis.

4. *Description of the gene for red eye*

The first red-eyed shrimp was found by Miss Jean Hanson in the fall of 1960, in the progeny of a brother-sister mating in the Utah stock. The data in Table IV indicate that the gene for red eye, *r*, is a recessive and has complete penetrance in the homozygote. Because the reciprocal crosses of the type $RR \times rr$ yield no red progeny, we may conclude that the gene is not sex-linked. Only 138/682, or 20%, of the F_2 shrimp had red eyes. The deviation from the expected 25% is highly significant ($P < 0.01$) and suggests that the red-eyed shrimp may have a lower viability than that of the black-eyed shrimp. The data from the ten backcross ($Rr \times rr$) matings also suggest a lower viability of the red-eye phenotype although the deviation from the expected 1 : 1 ratio is not significant ($P = 0.50$–0.30). Because the backcross data favor a single factor hypothesis, we may conclude that the red eye characteristic is governed by one locus. The three phenotypes may be described in this manner:

RR (black). The ocellus is pale red in the first instar of Heath (1924), black in subsequent instars. The two lateral eyes are black from the time they are first pigmented in the third instar and remain black throughout the life of all wild type shrimp.

Rr (black). The ocellus is pale red in the first instar, black in subsequent instars. The eyes are black throughout the life of most *Rr* shrimp. In rare instances, the eyes turn a deep ruby for a few days in the second week of life but revert to black.

rr (red). The eyes and ocellus are pale red for the first ten days; the pigment is so sparsely distributed throughout the first five instars that the three pigmented areas cannot be seen when the metanauplius is examined under the binocular microscope (7 ×). At the end of the second week, the eyes and ocellus are bright red. The eyes darken progressively after the shrimp has reached sexual maturity; by the twenty-second day, the eyes are dark ruby or black. The ocellus remains red for a longer period but may also turn ruby or black.

5. *Test for pseudogamy*

Pseudogamy is defined as the development of an egg parthenogenetically after the initial stimulus of penetration by a sperm. (The sperm nucleus then degenerates and has no effect on the genotype of the offspring.) Because the 39

TABLE IV

Segregation of the gene r *in the Utah race*

Number of matings	Mating	Progeny		
		Black	Red	Total
56	$rr \times rr$	0	1793	1793
10	$Rr \times rr$	161	149	310
32	$Rr \times Rr$	544	138	682
39	$\male RR \times \female rr$	720	0	720
12	$\male rr \times \female RR$	236	0	236

matings of the type $\male RR \times \female rr$ (listed in Table IV) produced only black-eyed offspring, we may conclude that pseudogamy is not the normal form of reproduction in Utah shrimp reared under standard laboratory conditions.

6. *The sequence of events in the reproductive cycle*

The reproductive system of the female consists of two ovaries, two pouch-like oviducts, and a ventral median uterus. The following events normally take place in a 24- to 48-hour period. The female expels from the uterus the first egg generation (brood A) as either virgin eggs or nauplii. The birth process takes from two to ten hours. She molts in a few seconds and then the next egg generation (brood B) passes from the ovaries into the oviducts in less than two hours. They remain there from one to 40 hours, whether copulation occurs or not. They then pass into the uterus, the process taking less than 30 minutes.

The eggs remain in the uterus for three to five days, irrespective of whether or not they are fertilized. The cycle is normally completed in from four to six days. However, in three exceptional females, the eggs lodged in the oviducts for ten days and the cycle was prolonged.

Lochhead (1941) correctly stated this sequence of events but did not publish the evidence for his conclusion that copulation occurred when the eggs were in the oviducts. Therefore, nine *rr* females were successively mated to males of *rr, RR,* and *rr* genotypes but the *RR* male was present only at the time when the eggs were seen to be in the oviducts. The *RR* males often failed to clasp during this short period and the females then laid eggs. Nauplii were obtained from three females; observations on one of them are in Table V. In all three cases, the *RR* male was present only during the time when the eggs were in the oviducts yet all the progeny were of the *Rr* genotype. Fautrez-Firlefyn (1957) and Goldschmidt (1952) have reported that eggs in the oviducts are in metaphase of the first meiotic division.

TABLE V

Observations on the reproductive cycle of one rr *female*

Duration of period	Events
4 days	The first male (*rr*) clasps the female. Egg generation A undergoes segmentation in the uterus. Egg generation B becomes visible in the ovaries due to accumulation of opaque yolk.
10 hours	70 nauplii (brood A) are expelled from the uterus. The *rr* male continues to clasp and attempts unsuccessfully to copulate. The female molts. The clasping pair is transferred to a slide and the male is pulled away. The female is returned to the vial.
55 minutes	Egg generation B passes into the oviducts. The second male (*RR*) is added. He clasps and copulates. Afterward, one seminal vesicle is transparent; the other is opaque due to the presence of sperm. The clasping pair is transferred to a slide and the male pulled away. The female is returned to the vial. Egg generation B passes into the uterus.
4 days	The third male (*rr*) is added. He clasps the female within twenty minutes after the eggs have entered the uterus. Four days later, 115 nauplii (brood B) are born.

58 nauplii of brood A survive to an age when they can be classified. All have red eyes. The 98 survivors of brood B have black eyes.

7. *Studies of the female reproductive cycle with tests for sperm storage*

Observations on more than 200 females of each race indicated that if they mated once, they produced a single brood of nauplii and thereafter laid thin-shelled eggs which did not hatch. This suggests that *Artemia* females do not store sperm as do *Drosophila* females. However, this evidence is not conclusive because the acts of clasping or copulation might in some way be essential for egg maturation. (For example, copulation might be the stimulus needed to bring about the reflex secretion by the oviduct of a substance which would cause the eggs to complete the first meiotic division.) This possibility is remote but, if true, it would invalidate the previously described tests for parthenogenesis as well as those for sperm storage. Therefore, the following experiments were designed to rule out this

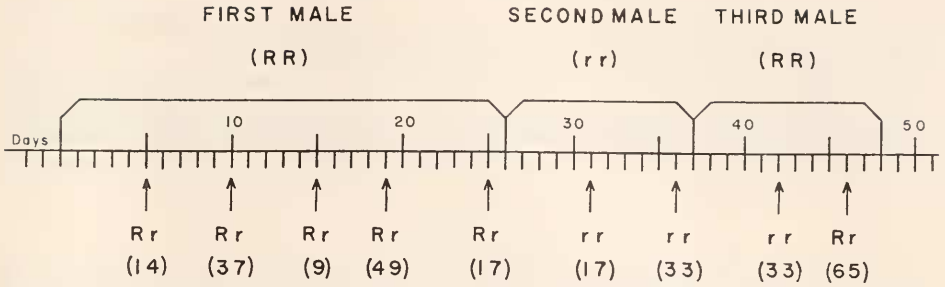

FIGURE 1. Genotypes of nine successive broods of nauplii produced by one *rr* Utah female mated to three different males. The numbers in parentheses indicate the number of progeny in each brood which survived to an age when their eye color could be classified.

possibility and to test for parthenogenesis and for sperm storage in a female which was at all times in the presence of a male.

RR males were hatched from cysts collected from Great Salt Lake; *rr* shrimp were selected from the Utah laboratory cultures. Twenty *rr* females were studied; each was alternately mated to males of *RR* and *rr* genotypes. The record of one of these females is seen in Figure 1. On the twenty-fifth day, a brood of *Rr* nauplii was produced as the result of a mating with an *RR* male. On the twenty-sixth day, the first male was removed, a second male with *rr* genotype was added, the eggs moved into the oviducts, and copulation occurred. These observations on the transparent female are confirmed by the fact that the nauplii born on the thirty-first day had the *rr* genotype. On the thirty-sixth day a brood of *rr* nauplii was born, the next generation of eggs passed into the oviducts, and the female again mated with the second male. On the thirty-seventh day, a third male was added, he attempted to copulate, but was unable to affect the genotype of the next brood because the eggs were now in the uterus.

In Figure 1, two changes in brood paternity may be seen: one between the twenty-fifth and thirty-first days and another between the forty-second and forty-sixth days. Another six changes in brood paternity are summarized in Table VI; in each case the two broods (A and B) are less than six days apart. Note that in the second (B) brood, all the nauplii have the same genotype because sperm are not stored by the female from one cycle to the next. Corroborative data were obtained from the other females, but in each case a brood of virgin eggs

TABLE VI

Comparison of pairs of broods of different paternity born to six rr *females*

Brood	Number of nauplii classified and their genotype					
	Female					
	1	2	3	4	5	6
A	73 *Rr*	30 *rr*	31 *Rr*	58 *Rr*	17 *rr*	22 *rr*
B	56 *rr*	46 *Rr*	41 *rr*	33 *rr*	26 *Rr*	12 *Rr*

separated the two broods of different paternity because the second male failed to clasp in the short period when the eggs were in the oviducts.

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Summary

1. This paper reports the first analysis of an inherited trait governed by one locus in the brine shrimp, *Artemia salina*. The autosomal gene, *r*, for red eyes arose spontaneously in a Utah race. It is recessive to the wild type allele, *R*, for black eyes. It has complete penetrance in *rr* shrimp.

2. The standard culture method outlined here has successfully carried the mutant stock through ten generations in a one-year period.

3. Reproduction was studied in two races from California and Utah. Neither paedogenesis nor parthenogenesis was observed in these shrimp which were raised by the standard culture method. This observation conflicts with the reports of Jensen and of Relyea that the Utah race could reproduce parthenogenetically.

4. Matings of *RR* males to *rr* females produce only black-eyed progeny. This indicates that when raised by the standard method the Utah shrimp do not normally reproduce by pseudogamy.

5. Studies of the sequence of steps in the female reproductive cycle confirm the observations of Lochhead. Genetic experiments have demonstrated that although the adults may clasp continuously throughout the cycle, copulation is effective only when the eggs are in the oviducts.

6. Females do not store sperm from one reproductive cycle to the next. If an *rr* female is alternately mated in different cycles to males of *RR* and *rr* genotype, all the nauplii in one brood have the same genotype.

LITERATURE CITED

Barigozzi, C., 1957, Différenciation des génotypes et distribution géographique d'*Artemia salina* Leach: données et problèmes. *Ann. Biol.*, **33**: 241–250.

Carpelan, L. H., 1957. Hydrobiology of the Alviso salt ponds. *Ecology*, **38**: 375–390.

Fautrez-Firlefyn, N., 1957. Protéines lipides et glucides dans l'oeuf d'*Artemia salina*. *Arch. Biol.*, **68**: 249–296.

Goldschmidt, E., 1952. Fluctuation in chromosome number in *Artemia salina*. *J. Morph.*, **91**: 111–133.

Heath, H., 1924. The external development of certain phyllopods. *J. Morph.*, **38**: 453–483.

Jensen, A. C., 1918. Some observations on *Artemia gracilis*, the brine shrimp of Great Salt Lake. *Biol. Bull.*, **34**: 18–32.

Lochhead, J. H., 1941. *Artemia*, the "brine shrimp." *Turtox News*, **19**: 41–45.

Lochhead, J. H., 1950. *Artemia*. *In:* Selected Invertebrate Types (pp. 394–399). Edited by F. A. Brown, Jr. John Wiley and Sons, Inc., New York.

Provasoli, L., and K. Shiraishi, 1959. Axenic cultivation of the brine shrimp *Artemia salina*. *Biol. Bull.*, **117**: 347–355.

Relyea, G. M., 1937. The brine shrimp of Great Salt Lake. *Amer. Nat.*, **71**: 612–616.

Weisz, P. B., 1946. The space-time pattern of segment formation in *Artemia salina*. *Biol. Bull.*, **91**: 119–140.

Weisz, P. B., 1947. The histological pattern of metameric development in *Artemia salina*. *J. Morph.*, **81**: 45–95.